

Neurotrophins and Time: Different Roles for TrkB Signaling in Hippocampal Long-Term Potentiation

Hyejin Kang,* Andy A. Welcher,[†] David Shelton,[‡]
and Erin M. Schuman*[§]

*Howard Hughes Medical Institute
Division of Biology 216–76
California Institute of Technology
Pasadena, California 91125

[†]Department of Immunology
Amgen Center 5-1-E
Amgen, Incorporated
Thousand Oaks, California 91320

[‡]Department of Neuroscience
Genentech, Incorporated
South San Francisco, California 94080

Summary

We examined the role of TrkB ligands in hippocampal long-term potentiation (LTP) using function-blocking TrkB antiserum (Ab) and Trk-IgG fusion proteins. Incubation of hippocampal slices with TrkB Ab had no effect on basal synaptic transmission, short-term plasticity, or LTP induced by several trains of tetanic stimulation. The TrkB Ab-treated slices, however, showed significant deficits in LTP induced by either theta-burst stimulation (TBS) or “pairing.” Slices exposed to the same number of inducing stimuli, delivered either as TBS or as a single 100 Hz epoch, only exhibited TrkB-sensitive LTP when TBS was used, indicating that the temporal pattern of stimulation determines the neurotrophin dependence. The late phase of LTP (2–3 hr) was also significantly impaired in slices pretreated with TrkB Ab or a Trk-IgG. The application of a Trk-IgG 30 min after LTP induction caused previously potentiated synaptic transmission to return to baseline levels, indicating that TrkB ligands are required to maintain LTP for up to 1 hr after induction. Taken together, these results indicate that both the temporal patterns of synaptic activity and the different temporal phases of synaptic enhancement are important in determining the neurotrophin dependence of plasticity in the hippocampus.

Introduction

The neurotrophic factors, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5), have recently gained attention as signaling molecules in both developmental and adult synaptic plasticity (reviewed by Lo, 1995; Thoenen, 1995; Bonhoeffer, 1996; Berninger and Poo, 1996). The persistent expression of the neurotrophins and their receptors in the adult hippocampus (Ernfors et al., 1990; Klein et al., 1990; Lamballe et al., 1991) suggests that these molecules may be involved

in activity-dependent neuronal plasticity. Indeed, neuronal activity can regulate neurotrophin expression: activation of excitatory or inhibitory receptors up-regulates or down-regulates, respectively, the synthesis of neurotrophins (Zafra et al., 1991; Gwag and Springer, 1993; Knipper et al., 1994; Wetmore et al., 1994). Experimentally induced seizure or injury can also induce a marked increase in neurotrophin mRNA levels (Ballarin et al., 1991; Isackson et al., 1991).

In addition to the above studies, there is also evidence that neurotrophins are capable of modulating synaptic transmission on a much faster timescale. The activity-dependent release of NGF and BDNF from hippocampal neurons has recently been reported (Bloch and Thoenen, 1995; Griesbeck et al., 1995, Soc. Neurosci., abstract; Goodman et al., 1996), suggesting that neurotrophins may modulate synaptic function. Indeed, the extracellular application of BDNF or NT-3 can enhance excitatory synaptic transmission (Lessmann et al., 1994; Kang and Schuman, 1995; Levine et al., 1995; Kang et al., 1996; Carmignoto et al., 1997) and inhibit GABAergic transmission (Kim et al., 1994). Several recent studies have begun to address whether endogenous TrkB ligands play a role in long-term potentiation (LTP), a widely studied model of synaptic plasticity. The earliest evidence suggesting a link between neurotrophic factors and hippocampal LTP was the demonstration that both BDNF and NT-3 mRNA are up-regulated by high frequency stimulation used to induce LTP (Patterson et al., 1992). Two different lines of mice with a targeted disruption of the BDNF gene exhibit a significant impairment in hippocampal LTP (Korte et al., 1995; Patterson et al., 1996), although only one line displays deficits in basal synaptic transmission (Patterson et al., 1996). Importantly, the deficits in LTP in BDNF knock-out mice can be rescued by acute treatment with either exogenous BDNF (Patterson et al., 1996) or an adenovirus construct containing the BDNF gene (Korte et al., 1996), excluding the possibility that the observed LTP deficits are due to developmental abnormalities. Figurov et al. (1996) have also recently demonstrated that exogenous BDNF promotes the induction of LTP in young hippocampal slices by tetanic stimulation that normally gives rise to only short-term potentiation. In addition, preventing TrkB activation with Trk-IgG fusion proteins greatly reduces the magnitude of LTP induced by theta-burst stimulation (TBS) in slices from adult hippocampus (Figurov et al., 1996). Taken together, these data support the view that BDNF or other TrkB ligand(s) play an active role in the expression of hippocampal LTP.

In this report, we examine the functional involvement of TrkB ligands in both early (1 hr) and late-phase (2–3 hr) LTP, using antisera that recognize and block TrkB function (Kang et al., 1996; Yan et al., 1997) as well as Trk-IgG fusion proteins that bind and inhibit endogenous TrkB ligands (Shelton et al., 1995). We find a differential effect of inhibiting TrkB function on LTP: some types of LTP-inducing stimuli produce TrkB-sensitive LTP, whereas others do not. These observations suggest that different temporal patterns of plasticity-inducing synaptic stimulation may invoke, at least in part,

[§]To whom correspondence should be addressed.

distinct cellular signaling mechanisms. For late-phase LTP, TrkB function may play a role in the late, protein synthesis-dependent phase of synaptic enhancement, which is consistent with previous reports demonstrating neurotrophin-induced protein synthesis and gene expression in brain (Ip et al., 1993; Ginty et al., 1994; Nawa et al., 1994; Kang and Schuman, 1996).

Results

TrkB Antisera Prevent TrkB Signaling

We previously described a rabbit polyclonal antiserum that specifically recognizes the TrkB receptor (Yan et al., 1997). As this antiserum was generated against a nondenatured antigen produced in a mammalian cell line, it should recognize correctly folded, glycosylated TrkB protein. The ability of the antiserum to block the BDNF-induced phosphorylation of the TrkB receptor was first tested using recombinant NIH3T3 cells, which express the full-length TrkB protein. The cells were coincubated with antiserum in the presence or absence of 50 ng/ml BDNF, and phosphorylation of the TrkB receptor was examined. As shown in the upper panel of Figure 1A, BDNF induced a strong phosphorylation of the TrkB receptor in cells coincubated with either media alone or media containing 10% preimmune serum. When cells were treated with a mixture of BDNF and media containing 10% anti-TrkB antisera, however, TrkB phosphorylation was completely blocked. In order to investigate the mechanism of the antiserum's action, we examined the effect of antiserum treatment on the levels of TrkB protein by probing similar Western blots with the anti-Trk antiserum. Treatment of NIH3T3 cells with the anti-TrkB antiserum for 1 hr resulted in a profound decrease in the amount of TrkB protein to levels below detection (Figure 1A, bottom). Similar results were observed with or without cotreatment of BDNF with the antiserum. In contrast, treatment without antiserum or treatment with preimmune serum had no effect on the levels of TrkB protein in the cells. The slightly reduced levels of protein seen in the BDNF-treated samples likely result from ligand-induced internalization and degradation, phenomena commonly observed with ligand treatment of many receptor protein tyrosine kinases.

Previous reports have shown that antibodies against the Trk receptors can act as either agonists or antagonists (Clary et al., 1994; LeSauter et al., 1996). Our results described above indicated that 1 hr of antisera pretreatment can block TrkB function. However, it was possible that at shorter pretreatment times, the antisera might act as an agonist. To address this possibility, we treated slices with the TrkB antiserum for 0, 15, or 60 min and examined the extent of TrkB phosphorylation following immunoprecipitation and Western blot analysis. We found that the amount of tyrosine phosphorylation of TrkB was not increased by antibody treatment, even at shorter time points, indicating that the TrkB antiserum does not act as an agonist in our experiments (Figure 1B). Although the exact mechanism of antisera-induced TrkB receptor inactivation in hippocampal slices is unknown, it is likely to be similar to that seen in the treated NIH3T3 cells. We previously demonstrated that

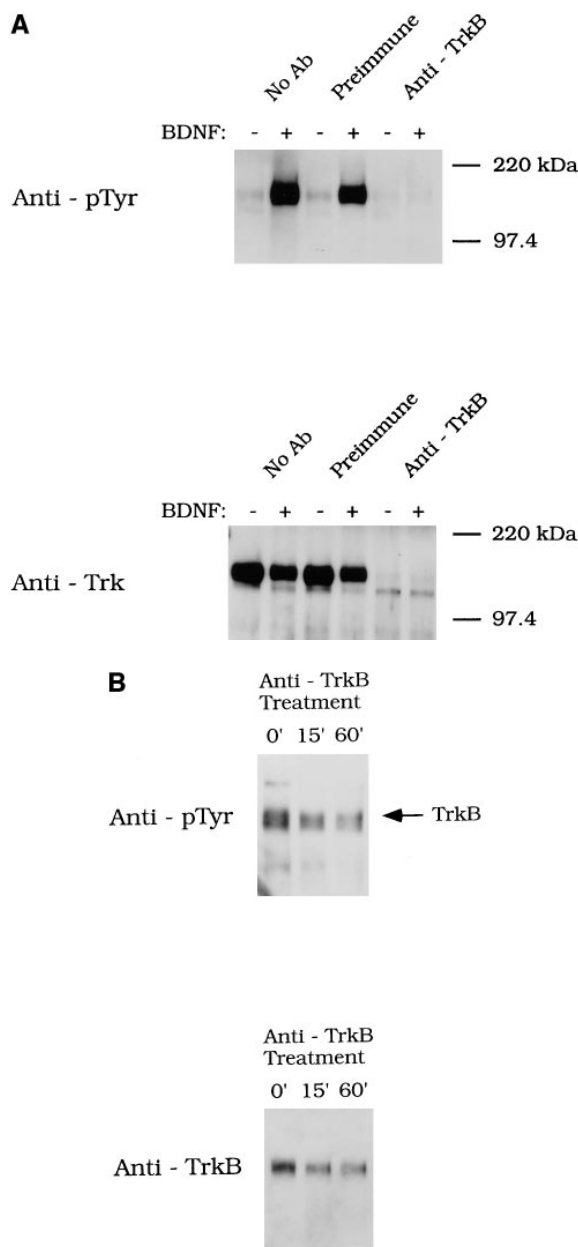


Figure 1. Characterization of Anti-TrkB Antisera

(A) Anti-TrkB antisera block BDNF-induced TrkB phosphorylation in NIH3T3 cells. Recombinant NIH3T3 cells expressing the full-length TrkB receptor were incubated with media lacking antisera, media containing preimmune sera, or media containing anti-TrkB antisera, in the absence (–) or presence (+) of BDNF. Immunoprecipitated Trk receptors from treated cells were examined by Western blot analysis. The blots were probed with either anti-pTyr antisera (top) or anti-Trk antisera (bottom).

(B) Treatment with anti-TrkB antisera does not induce TrkB phosphorylation in hippocampal slices. Slices were incubated with anti-TrkB antisera for 0, 15, or 60 min, and immunoprecipitated Trk receptors from treated slices were examined by Western blot analysis. The blots were probed with either anti-pTyr antisera (top) or anti-TrkB antisera (bottom).

treatment of hippocampal slices with this anti-TrkB antiserum completely prevents the synaptic enhancement produced by exogenous application of BDNF (Kang et

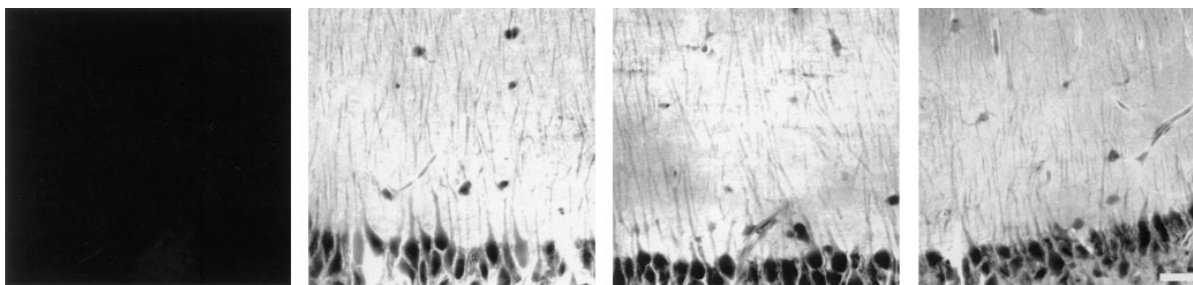


Figure 2. TrkB Ab Can Penetrate Hippocampal Slices and Persist following Perfusion with ACSF

Shown are 63 \times confocal images taken from the middle 150–200 μ m of a hippocampal slice treated with a secondary antibody to recognize the TrkB Ab. From left to right are sections from slices treated with no primary antibody (dark image on left) or a TrkB Ab perfused with normal ACSF for 0, 30, or 60 min. Scale bar, 25 μ m.

al., 1996). Using the same protocol to introduce the antibody into hippocampal slices, we have now examined the effects of blocking TrkB function on basal synaptic transmission and short- and long-term synaptic plasticity.

Function-Blocking TrkB Antiserum Does Not Affect Basic Synaptic Function

To ascertain the specificity of the function-blocking TrkB antiserum (Ab) on LTP, we first examined several aspects of basic synaptic function at Schaffer collateral–CA1 synapses in the hippocampal slice. We confirmed both the successful penetration of the Ab into hippocampal slices and the persistence of the antibody following perfusion with normal artificial cerebrospinal fluid (ACSF; Figure 2). Basal synaptic transmission was examined in anti-TrkB treated slices by generating input–output curves of synaptic strength. We plotted stimulus strength against the slope of the field excitatory postsynaptic potential (field EPSP) to compare the size of the response for a given intensity of stimulus in preimmune serum or TrkB Ab-incubated slices (Figure 3A). For all of the stimulus strengths tested, equivalent synaptic responses were evoked for both the control and the anti-TrkB treated groups. Dividing the slope of the field EPSP by the size of the presynaptic fiber volley (PSFV) provides a measure of the synaptic response size produced by stimulation of a given number of presynaptic axons. We found no significant difference in either the mean or the distribution of these values between preimmune and TrkB Ab-incubated slices (Figure 3B). Similar measurements were obtained from slices treated with Ab for >3 hr (data not shown). These data suggest that basal synaptic transmission is not modified by acute manipulation of TrkB function.

We next examined two forms of short-term synaptic plasticity, paired-pulse facilitation (PPF) and posttetanic potentiation (PTP). Both PPF and PTP reflect the plasticity of presynaptic processes; the larger synaptic responses observed following stimulation are due to enhanced neurotransmitter release via an accumulation of Ca^{2+} in the presynaptic nerve terminal (Katz and Miledi, 1968; Delaney et al., 1989). The TrkB Ab had no distinguishable effect on the size of PTP (Figure 4; preimmune, 345.8% \pm 48.7%; TrkB Ab, 314.3% \pm 13.6%; n = 9) or PPF (Figure 3C; 100 ms delay: preimmune, 129.3% \pm

4.2%; TrkB Ab, 124.9% \pm 3.1%; n = 16). Similar measurements were obtained in slices treated with Ab for >3 hr (data not shown). These results suggest that pre-synaptic release processes are unperturbed by acute manipulation of TrkB function.

LTP-Inducing Stimuli Determine TrkB Sensitivity

Long-term potentiation of excitatory synaptic transmission can be induced by a variety of different stimulation protocols such as high frequency stimulation (tetanus), TBS, or pairing postsynaptic depolarization with low frequency stimulation (pairing; Bliss and Lomo, 1973; Larson and Lynch, 1986; Gustafsson et al., 1987). Potentiation induced by each of these methods requires the activation of N-methyl-D-aspartate (NMDA) receptors and postsynaptic Ca^{2+} during LTP induction. We examined whether TrkB receptor signaling plays a role in the induction of LTP induced by different stimulation protocols including 100 Hz stimulation, TBS, and pairing. At the highest frequency stimulation protocol examined, four trains of 100 Hz (1 s) tetani, treatment with the TrkB Ab did not affect either the induction or the maintenance of LTP (Figure 4A; mean percent of baseline at 50–60 min: preimmune, 186.0% \pm 11.9%; n = 9, p < 0.001; TrkB Ab, 170.2% \pm 11.9%; n = 9, p < 0.005). (This lack of inhibition is consistent with our previous observation [Kang and Schuman, 1995] that preexposure of hippocampal slices to BDNF does not occlude subsequent attempts to induce LTP by four trains of 100 Hz stimulation.) When we induced LTP by patterned (TBS) or low frequency (pairing) stimulation protocols, however, TrkB Ab-incubated slices showed significant deficits in LTP (Figures 4B and 4C; mean percent of baseline at 50–60 min, TBS: preimmune, 137.7% \pm 6.0%; n = 9, p < 0.001; TrkB Ab, 112.0% \pm 5.5%; n = 9, p < 0.05; pairing: preimmune, 167.3% \pm 15.9%; n = 9, p < 0.001; TrkB Ab, 108.0% \pm 9.1%; n = 9, not significant [NS]). Pretreatment of hippocampal slices with K252a (200 nM), an inhibitor of receptor tyrosine kinases (Tapley et al., 1992), inhibited TBS-induced LTP to a similar extent as the TrkB Ab (mean percent of baseline at 50–60 min: control, 135.6% \pm 3.8%; n = 3, p \leq 0.05; K252a, 106.8% \pm 2.8%; n = 5, NS). A 10-fold lower dilution of TrkB Ab was ineffective in preventing TBS-induced LTP, whereas a \sim 1.5-fold higher dilution produced no greater inhibition (mean percent of baseline at 50–60 min: TrkB

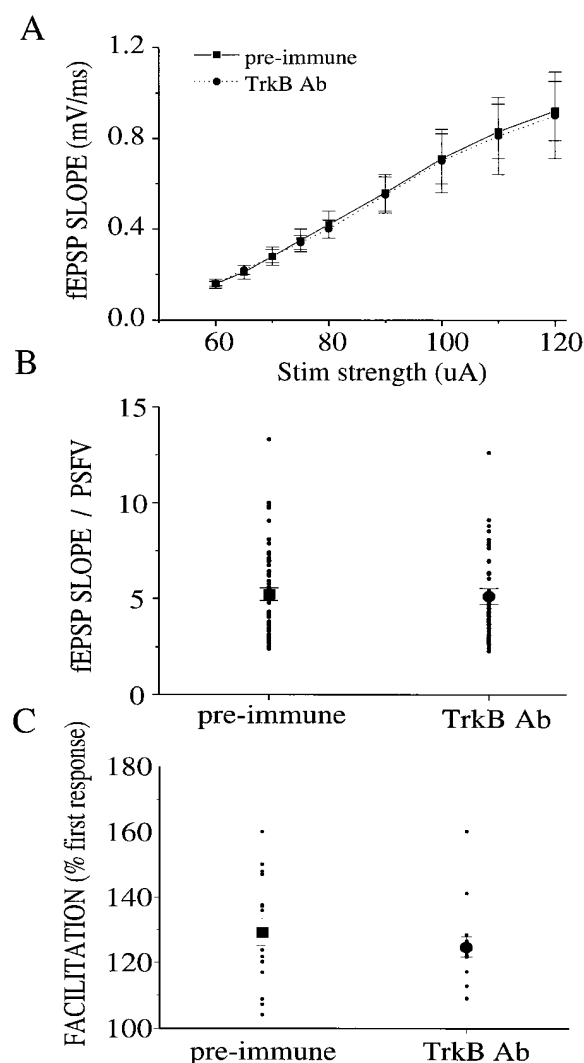


Figure 3. Basal Synaptic Transmission and Paired-Pulse Facilitation (PPF) Are Normal in Slices Pretreated with Function-Blocking TrkB Antisera

In (A) through (C), the control and TrkB antisera groups are not significantly different from one another.

(A) Input-output curves plotting stimulus strength against the initial slope of the field EPSP in preimmune serum- (squares; solid line) and TrkB antisera- (circles; dotted line) treated slices ($n = 7$). (B) Scatter plot depicting the ratio of the field EPSP slope to the presynaptic fiber volley (PSFV) amplitude over a range of stimulus strengths ($n = 7$). For each group the mean \pm SEM is indicated by the large symbol with the error bars.

(C) Scatter plot depicting the facilitation ratios (slope of second EPSP/slope of first EPSP) obtained with an interstimulus interval of 100 ms ($n = 16$). For each group, the mean \pm SEM is indicated by the large symbol with the error bars.

Ab, 1:100 dilution, $137.3\% \pm 7.2\%$; $n = 3$, $p \leq 0.05$; TrkB Ab, 1:7 dilution, $102.4\% \pm 6.7\%$; $n = 3$, NS).

If TrkB signaling is important for LTP induced by TBS, then prior exposure of hippocampal slices to a TrkB ligand might be expected to attenuate the amount of LTP elicited by subsequent TBS. Indeed, we found that

prior potentiation of synaptic transmission by BDNF application significantly reduced the amount of LTP subsequently elicited by TBS (Figure 5; mean percent of baseline at 50–60 min: $108.7\% \pm 10.2\%$; $n = 6$, NS). Taken together, these data suggest that the TrkB receptor, activated by BDNF or other TrkB ligands, is required for LTP induced by TBS or by pairing. Potentiation induced by several trains of high frequency stimulation, however, does not appear to require TrkB function, suggesting that neurotrophin-mediated signaling pathways may not be activated during this particular protocol or that compensatory mechanisms exist that obviate the need for TrkB function.

The Importance of Synaptic Activity Patterns

In the above experiments, there are several differences in the stimulation protocols used, including the frequency of stimulation, the pattern of stimulation, and the number of times a given stimulation protocol was applied (e.g., one epoch of TBS versus four epochs of 100 Hz stimulation). Any one or a combination of these variables could determine the TrkB dependence or independence of the synaptic enhancement. It is possible, for example, that increasing the number of times a TBS protocol is applied to induce LTP may confer TrkB independence to the synaptic enhancement. Accordingly, we tested this in TrkB Ab-treated slices and found that LTP was still sensitive to TrkB inhibition when multiple (three) TBS epochs were used as inducing stimuli (Figure 6; mean percent of baseline at 50–60 min: preimmune, $139.1\% \pm 11.5\%$; $n = 7$, $p \leq 0.005$; TrkB Ab, $110.4\% \pm 8.8\%$; $n = 7$, NS). The inhibition of LTP observed in TrkB Ab-treated slices exposed to three epochs of TBS was not significantly different from that observed in slices exposed to a single epoch of TBS. These results indicate that increasing the number of exposures to TBS does not alter the dependence of LTP on TrkB function.

We next investigated whether the particular pattern of stimulation used to induce LTP is a relevant variable. To do this, we applied a stimulation protocol that included the same number of pulses as three epochs of TBS (120 pulses total) delivered in a different pattern (100 Hz for 1.2 s). Interestingly, the TrkB Ab-treated slices exposed to this LTP induction protocol did not show diminished LTP when compared to preimmune controls (Figure 6; mean percent of baseline at 50–60 min: preimmune, $153.9\% \pm 9.4\%$; $n = 8$, $p \leq 0.005$; TrkB Ab, $155.4\% \pm 5.8\%$; $n = 7$, $p \leq 0.005$). Thus, an equivalent number of pulses, delivered either as a single 100 Hz epoch or as several bursts of stimuli at the theta frequency, can produce LTP that is either independent or dependent on TrkB function. In addition, it should be noted that the interstimulus interval within an individual theta burst is the same as that in the 100 Hz paradigm (100 Hz; Figure 6); this indicates the importance of the macroscopic, rather than the microscopic, temporal pattern in determining the TrkB dependence of the potentiation.

TrkB Signaling in Late-Phase LTP

Long-lasting forms of synaptic plasticity induced by high frequency stimulation require new protein synthesis

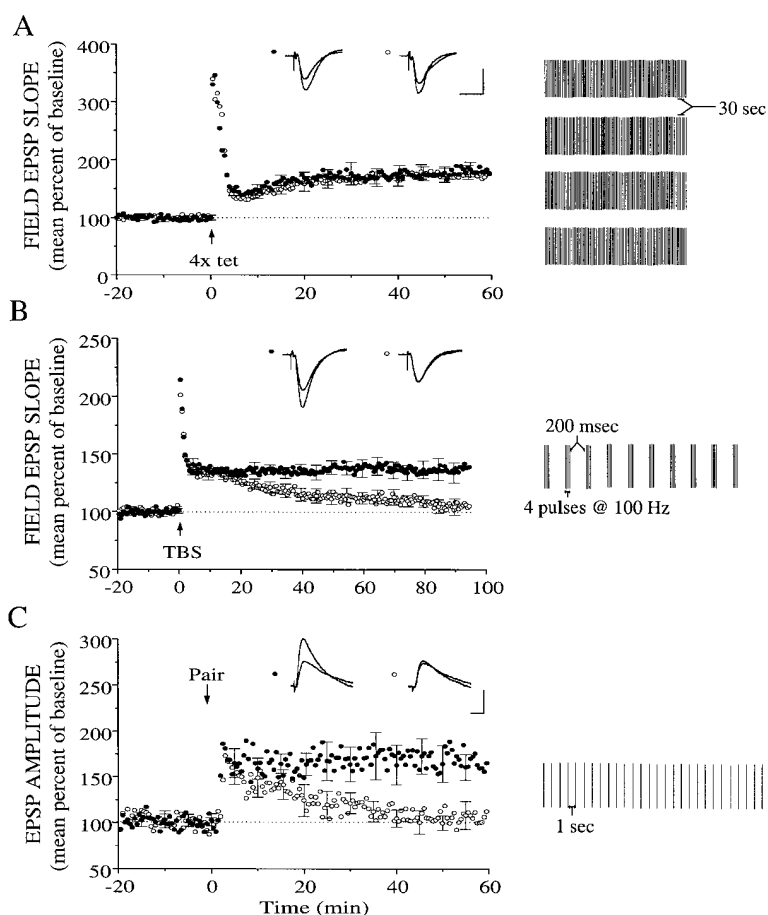


Figure 4. Effects of Function-Blocking TrkB Antisera on LTP Induced by Different Protocols

Ensemble averages showing mean percent of baseline values of field EPSP initial slopes (A) and [B]) or intracellular EPSP amplitudes (C) before and after LTP induction in slices pretreated with either preimmune serum (closed circles) or TrkB Ab (open circles). In each panel, superimposed representative EPSPs shown were recorded 5 min before and 1 hr after LTP induction; $n = 9$ for each control and experimental group below. Scale bars, 1 mV and 20 ms ([A] and [B]); 5 mV and 20 ms (C).

(A) LTP induced by four trains of tetanic stimulation (one train = 100 Hz for 1 s). Mean field EPSP slope was 0.12 ± 0.02 mV/ms (mean \pm SEM) before and 0.21 ± 0.02 mV/ms after LTP induction in preimmune serum-treated slices and 0.15 ± 0.01 mV/ms before and 0.25 ± 0.03 mV/ms after LTP induction in TrkB Ab-treated slices.

(B) LTP induced by theta-burst stimulation (TBS; see Experimental Procedures). Mean field EPSP slope was 0.20 ± 0.01 mV/ms before and 0.27 ± 0.02 mV/ms after LTP induction in preimmune serum-treated slices and 0.18 ± 0.01 mV/ms before and 0.20 ± 0.01 mV/ms after LTP induction in TrkB Ab-treated slices.

(C) LTP induced by pairing postsynaptic depolarization with low frequency stimulation (see Experimental Procedures). Mean EPSP amplitude was 5.26 ± 0.38 mV before and 8.54 ± 0.55 mV after LTP induction in preimmune serum-treated slices, and 6.71 ± 0.50 mV before and 7.17 ± 0.67 mV after LTP induction in TrkB antibody-treated slices.

(Frey et al., 1988; Otani et al., 1989; Nguyen et al., 1994). Since neurotrophins can induce new protein synthesis and gene expression in a variety of systems (Ginty et al., 1994; Nawa et al., 1994), we examined the possible involvement of TrkB function in late-phase LTP. Long-term or late-phase LTP (L-LTP) was induced in the slice by three trains of high frequency stimulation separated by 5 min intervals (Frey et al., 1988). In contrast to control slices (incubated with preimmune serum), in which LTP persisted for up to 4 hr, TrkB Ab-incubated slices exhibited a slow decay of LTP over time, with synaptic responses completely returning to baseline levels within 3 hr of induction (Figures 7A and 7B; mean percent of baseline at 170–180 min: preimmune, $149.0 \pm 8.3\%$; $n = 8$, $p < 0.005$; TrkB Ab, $98.7 \pm 12.0\%$; $n = 8$, NS). The difference in the magnitude of LTP observed in TrkB-treated and preimmune control groups became significant ~ 1 hr after induction ($p < 0.05$), indicating that TrkB function is required to maintain the late phase of LTP. Similar results were obtained when an alternative method of blocking TrkB function, a TrkB-IgG, was employed. The TrkB-IgGs, composed of the extracellular portion of Trk receptors fused to the Fc domain of human IgG, are readily diffusible and serve as fast-acting competitive antagonists of functional Trk receptors by binding endogenous ligands (Shelton et al., 1995). Extracellular application of a TrkB-IgG ($1 \mu\text{g/ml}$) to hippocampal

slices 15 min prior to LTP induction resulted in a decaying potentiation that returned to baseline values within 150 min, without exerting any effect on synaptic transmission in a second control pathway within the same slice (Figure 7B; mean percent of baseline at 170–180 min: LTP pathway, $103.0 \pm 5.5\%$; $n = 3$, NS; control pathway, $97.7 \pm 5.6\%$; $n = 3$, NS). A twofold lower concentration of TrkB-IgG ($0.5 \mu\text{g/ml}$) had no inhibitory effect on L-LTP (mean percent of baseline at 170–180 min: LTP pathway, $152.3 \pm 8.8\%$; $n = 4$, $p < 0.05$; control pathway, $100.3 \pm 12.7\%$; $n = 4$, NS), whereas a fivefold higher concentration of TrkB-IgG ($5.0 \mu\text{g/ml}$) produced inhibition of L-LTP that was statistically indistinguishable from that observed with $1 \mu\text{g/ml}$ (mean percent of baseline at 170–180 min: LTP pathway, $116.2 \pm 9.3\%$; $n = 3$, NS; control pathway, $93.3 \pm 2.4\%$; $n = 3$, NS). In contrast, pretreatment of slices with a TrkA-IgG, which scavenges TrkA ligands such as NGF, had no effect on late-phase LTP (mean percent of baseline at 170–180 min: TrkA-IgG, $156.0 \pm 10.3\%$; $n = 3$, $p \leq 0.05$). Using a two-site ELISA technique (Cabelli et al., 1997), we determined that a similar percentage of TrkA and TrkB-IgG remained intact in hippocampal slices following perfusion with the TrkB-IgG for 30 min (percent intact: TrkA-IgG, $75.9 \pm 14.6\%$; TrkB-IgG, $82.9 \pm 9.5\%$).

The two sets of experiments described above indicate

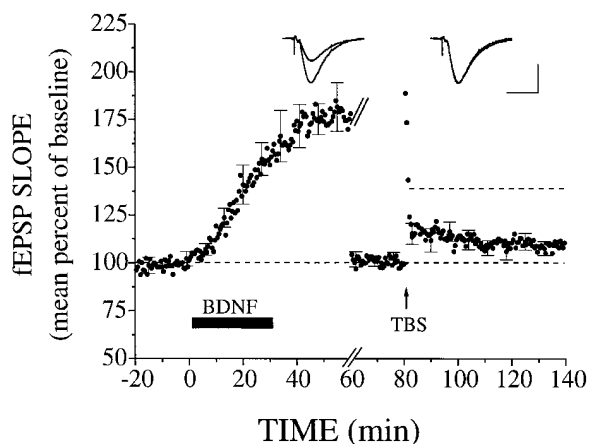


Figure 5. Prior Exposure to BDNF Reduces Subsequent TBS-Induced Potentiation

Ensemble average of six experiments showing mean percentage of baseline values of field EPSP slopes before and after application of BDNF (50 ng/ml) and before and after application of TBS. Superimposed traces shown are from 10 min before and 50–60 min after either BDNF application (right) or TBS (left). Scale bar, 1 mV/20 ms. The amount of potentiation elicited by TBS in these experiments was significantly less than that observed in control slices ($p \leq 0.05$; see Figure 4, indicated by dashed line) but not significantly different from TrkB Ab-treated slices (Figure 4). Mean field EPSP slope was 0.10 ± 0.02 mV/ms before and 0.17 ± 0.01 mV/ms 50–60 min after BDNF application. Mean field EPSP slope was 0.16 ± 0.01 mV/ms before and 0.18 ± 0.02 mV/ms 50–60 min after TBS.

that hippocampal slices exposed to either a function-blocking TrkB Ab or a TrkB-IgG prior to tetanic stimulation failed to exhibit L-LTP. Consistent with these observations, prior exposure of slices to BDNF also significantly attenuated both the magnitude and duration of late-phase LTP (Figure 8; mean percent of baseline at 170–180 min: $117.5\% \pm 11.3\%$; $n = 6$, NS).

The pattern of decaying potentiation evident in the TrkB-disabled slices is reminiscent of the potentiation kinetics observed when slices are tetanized in the presence of protein synthesis inhibitors (e.g., Frey et al., 1988). To test whether this truncation of LTP is due in part to the lack of neurotrophin-induced new protein synthesis (Kang and Schuman, 1996), we attempted to induce L-LTP in the presence of a protein synthesis inhibitor, anisomycin (40 μ M), alone and in combination with the function-blocking TrkB Ab. Treatment with anisomycin alone or anisomycin in combination with the TrkB Ab produced no further reduction in L-LTP when compared to TrkB Ab-only treated slices (Figures 8A and 8B; mean percent of baseline at 170–180 min: anisomycin alone, $115.5\% \pm 14.9\%$; $n = 7$, NS; TrkB Ab + anisomycin, $107.1\% \pm 10.7\%$; $n = 8$, NS), suggesting that TrkB signaling may contribute to the protein synthesis dependence of L-LTP.

What is the temporal window during which TrkB signaling is required for L-LTP? The slight reduction of LTP almost immediately after the tetanus in the above experiments indicates that neurotrophin signaling may be required around the time of induction. We next addressed whether neurotrophins signal exclusively during the induction of LTP by introducing Trk-IgG fusion

proteins well after the time associated with inductive signal transduction events. We conducted two-pathway experiments in which L-LTP was induced in one pathway while the other pathway, which received only the test stimuli, served as a control. TrkA-IgG or TrkB-IgG was introduced into the superfusate for 30 min, 30 min after LTP induction. The introduction of TrkA-IgG ($1 \mu\text{g ml}^{-1}$) was without effect on either the potentiated (Figure 9A) or the control pathway (Figure 9B; mean percent of baseline at 170–180 min: potentiated pathway, $166.8\% \pm 14.4\%$; $n = 6$, $p < 0.01$; control pathway, $90.1\% \pm 3.4\%$; $n = 5$, NS). Application of TrkB-IgG ($1 \mu\text{g ml}^{-1}$) 30 min after LTP induction, however, produced a reversal of LTP in the potentiated pathway (Figure 9C) without affecting synaptic transmission in the control pathway (Figure 9D; mean percent of baseline at 170–180 min: potentiated pathway, $107.4\% \pm 8.6\%$; $n = 7$, NS; control pathway, $104.5\% \pm 5.7\%$; $n = 6$, NS). These data suggest that the interaction of the TrkB receptor with its endogenous ligands is required to maintain potentiated synaptic transmission. Does continuous signaling by TrkB ligands underlie LTP maintenance for the entire duration of the synaptic enhancement? To address this, the TrkB-IgG was applied beginning 70 min after LTP induction. This delayed application of the TrkB-IgG had no effect on the previously established potentiation (Figure 9; mean percent of baseline at 170–180 min: $171.5\% \pm 18.2\%$; $n = 8$, $p \leq 0.005$). These data suggest that the critical temporal window during which TrkB signaling is required for L-LTP begins with induction and extends 30–60 min following the initiating synaptic stimuli.

Discussion

Several recent studies have supported the hypothesis that BDNF is involved in hippocampal LTP (Korte et al., 1995; Figurov et al., 1996; Korte et al., 1996; Patterson et al., 1996). We used two different specific inhibitors of TrkB function, a function-blocking TrkB antiserum and a TrkB-IgG fusion protein, to identify more specifically the role of TrkB and its ligands in synaptic plasticity induced by a variety of stimulation paradigms. Hippocampal slices treated with the TrkB antiserum exhibited normal basal synaptic transmission, paired-pulse facilitation, and posttetanic potentiation, consistent with results from one study of BDNF mutant mice (Korte et al., 1995; but see Patterson et al., 1996). The observation that synaptic transmission and short-term plasticity are intact in the absence of TrkB signaling suggests that the deficits in LTP in TrkB antiserum-treated slices are not due to disruption of normal synaptic processes. Moreover, two observations argue against the idea that the perturbations of TrkB function disable the synapse's ability to produce and detect the inducing stimuli: (1) at least one form of NMDA receptor-dependent LTP (4×100 Hz) can be elicited in the presence of the Ab (Figure 4), indicating that NMDA-dependent signaling is intact; and (2) pairing-inducing LTP is inhibited, indicating that the blockers cannot be working by preventing depolarization, as both control and Ab-treated slices were depolarized to an equivalent extent by intracellular current

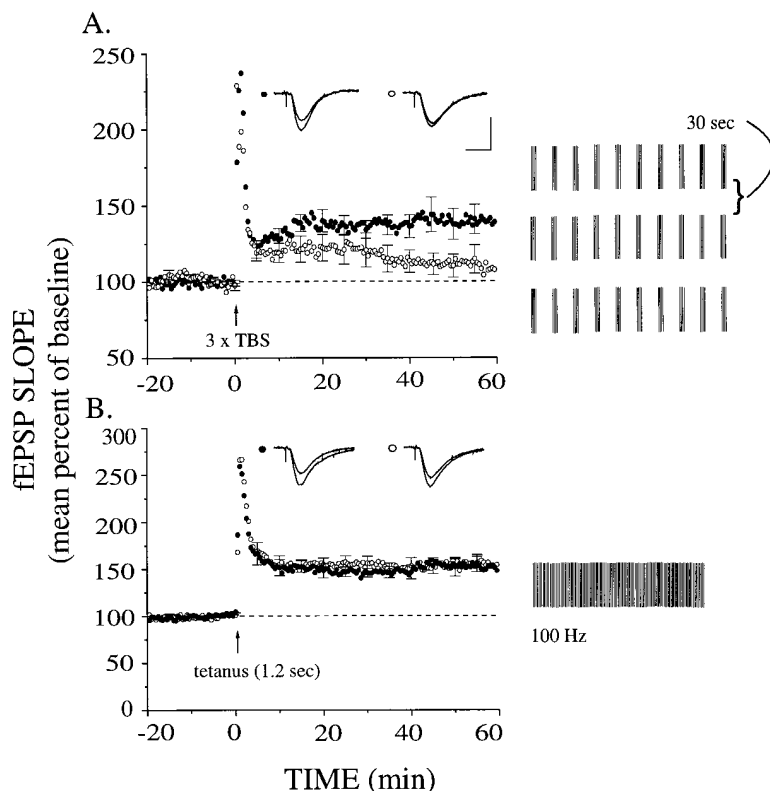


Figure 6. The Pattern of Stimulation, Not the Number of Pulses, Determines the TrkB Dependence of LTP

Superimposed traces shown are from 10 min before and 50–60 min after LTP induction. Scale bar, 1 mV/20 ms.

(A) Slices treated with TrkB Ab (open circles) show significantly diminished LTP in response to three epochs of TBS, relative to preimmune controls (closed circles; $n = 7$ for each). In control slices, the mean field EPSP slopes were 0.17 ± 0.01 mV/ms and 0.25 ± 0.03 mV/ms before and after TBS stimulation, respectively. In TrkB Ab-treated slices, the mean field EPSP slopes were 0.20 ± 0.01 mV/ms and 0.22 ± 0.03 mV/ms before and after TBS stimulation, respectively.

(B) Slices treated with TrkB Ab (open circles) show unimpaired LTP in response to a single epoch of 100 Hz stimulation, relative to preimmune controls (closed circles; $n = 7$ for TrkB Ab and 8 for controls). In control slices, the mean field EPSP slopes were 0.16 ± 0.01 mV/ms and 0.25 ± 0.01 mV/ms before and after 100 Hz stimulation, respectively. In TrkB Ab-treated slices, the mean field EPSP slopes were 0.16 ± 0.01 mV/ms and 0.25 ± 0.02 mV/ms before and after 100 Hz stimulation, respectively.

injection. A previous report indicated that TrkB-IgG reduced the postsynaptic response to repetitive stimulation (Figurov et al., 1996); we have not observed this under the experimental conditions used in this study (fourth EPSP/first EPSP ratio in a TBS burst: control, 30.3 ± 8.3 ; $n = 3$; TrkB IgG, 31.8 ± 4.9 ; $n = 3$).

Our data indicate that BDNF and/or other TrkB ligands play a critical role in both the early and the late-phase of synaptic potentiation in the adult rat hippocampus. Synaptic potentiation induced by bursts of stimuli delivered at the theta frequency or by pairing was significantly attenuated in TrkB Ab-treated slices. LTP induced by four trains of 100 Hz stimulation, however, was relatively insensitive to TrkB blockade. Moreover, occlusion experiments in this study and in a previous study (Kang and Schuman, 1995) are consistent with the idea that different induction protocols invoke different requirements for TrkB signaling. Prior treatment of hippocampal slices with BDNF significantly attenuates subsequent LTP induced by TBS (Figure 5) but not by four trains of 100 Hz stimulation (Kang and Schuman, 1995).

What particular feature of the various LTP induction protocols renders them more or less vulnerable to inhibition of BDNF-TrkB signaling? The different stimulation patterns used by us and others vary both in the absolute number of pulses given and in the pattern and/or frequency of synaptic stimulation. It appears that one crucial variable is the pattern of stimulation used to initiate the synaptic enhancement. A stimulation protocol consisting of 120 pulses delivered at a theta-like frequency revealed a dependence on TrkB function, whereas the same number of pulses delivered continuously at 100

Hz produced TrkB-independent LTP. There are at least two explanations to account for this observation: (1) different temporal patterns of stimulation can differentially promote the release of TrkB ligands, and (2) TrkB ligands are released under all stimulation protocols, but continuous, rather than burst-like, stimuli confer TrkB independence by recruiting additional biochemical signaling pathways. Regardless of the particular molecular explanation, these data indicate that hippocampal synapses are sensitive to the temporal pattern of their inputs and invoke different signaling pathways to produce synaptic enhancement, potentially reflecting the nature of the plasticity-inducing stimuli.

Thus, unlike many signal transduction events, including activation of NMDA receptors, rises in postsynaptic Ca^{2+} , and activation of Ca^{2+} calmodulin-dependent protein kinase II (CaMKII; Collingridge et al., 1983; Lynch et al., 1983; Malinow et al., 1989), which are essential for the induction of most forms of LTP in area CA1, the BDNF-TrkB signaling pathway does not appear to be necessary for LTP induced by all protocols. Continuous stimulation protocols, such as repeated trains of 100 Hz stimulation, may not favor release of TrkB ligands; alternatively, TrkB ligands may be released but not required for LTP, presumably due to compensation by alternative signaling pathways. The observed variable requirement for TrkB signaling as a function of stimulation pattern is true of other signaling molecules, such as nitric oxide (e.g., Haley et al., 1993; Williams et al., 1993; Schuman and Madison, 1994), and reinforces the notion that different LTP induction protocols involve different, although potentially overlapping, biochemical

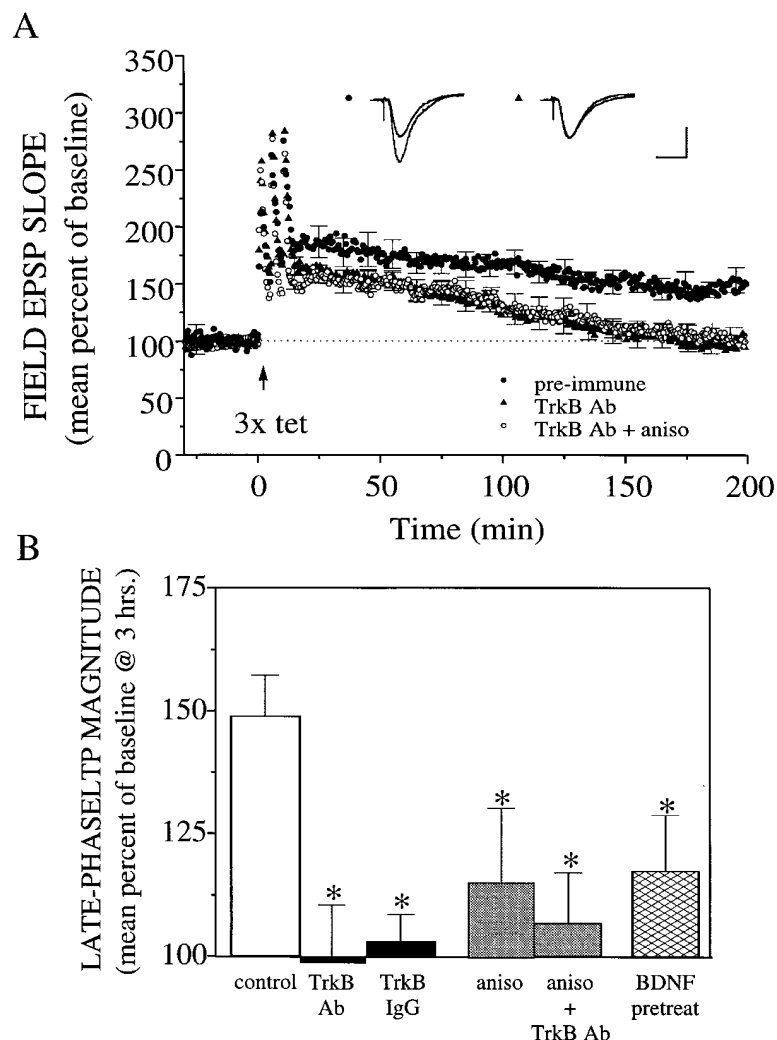


Figure 7. Attenuation of Late-Phase LTP by Function-Blocking TrkB Antisera and TrkB IgG

(A) Closed circles indicate the control LTP obtained in preimmune serum-treated slices ($n = 8$). Mean field EPSP slope was 0.16 ± 0.01 mV/ms (mean \pm SEM) before, 0.28 ± 0.02 mV/ms 1 hr after, and 0.23 ± 0.02 mV/ms 3 hr after LTP induction. Closed triangles indicate LTP obtained in TrkB antibody-treated slices ($n = 8$). Mean field EPSP slope was 0.19 ± 0.01 mV/ms before, 0.28 ± 0.02 mV/ms 1 hr after, and 0.18 ± 0.02 mV/ms 3 hr after LTP induction. Open circles indicate LTP obtained in the presence of TrkB Ab plus anisomycin ($n = 8$). Mean field EPSP slope was 0.18 ± 0.02 mV/ms before, 0.28 ± 0.03 mV/ms 1 hr after, and 0.19 ± 0.02 mV/ms 3 hr after LTP induction. Superimposed representative EPSPs were recorded 5 min before and 3 hr after LTP induction. Calibration bars, 1 mV and 20 ms.

(B) Summary of the percent potentiation of mean field EPSP slopes 170–180 min (3 hr) after LTP induction for various groups, as shown; * $p < 0.05$ versus control (preimmune) group.

processes. Whether the recruitment of distinct biochemical induction pathways results in different mechanisms of LTP expression (e.g., pre- versus postsynaptic) remains to be determined. As such, it is becoming increasingly clear that the acronym LTP describes enhanced synaptic transmission at a phenomenological level but cannot necessarily be taken to represent a singularity of signaling mechanisms.

The cellular mechanisms by which neurotrophins modulate synaptic plasticity are not well understood. BDNF application in cultured hippocampal neurons (Berninger et al., 1993) and nerve-muscle synapses (Stoop and Poo, 1996) dramatically increases intracellular Ca^{2+} . Our own studies indicate that Ca^{2+} influx through voltage-gated Ca^{2+} channels and intracellular Ca^{2+} stores is obligatory for the induction of BDNF-induced potentiation (Kang and Schuman, 1995, Soc. Neurosci., abstract). It is therefore possible that ligands of the TrkB receptor act together with other signaling pathways to regulate the level of intracellular Ca^{2+} upon neuronal stimulation. The magnitude of the Ca^{2+} rise may determine the degree to which Ca^{2+} activates downstream effectors (Gallin and Greenberg, 1995) and consequently influence the overall probability or magnitude of synaptic potentiation.

Our data demonstrating that treatment of slices with function-blocking TrkB antiserum or TrkB-IgG reduces late-phase LTP indicate a direct involvement of TrkB signaling in the maintenance of L-LTP. The kinetics and extent of LTP decline under these conditions is similar to the decay of LTP observed in the presence of protein synthesis inhibitors (Frey et al., 1988). Moreover, there was no additive inhibitory effect on LTP when slices were treated with both the TrkB antisera and the protein synthesis inhibitor anisomycin. This finding suggests that TrkB ligands released during LTP may participate in stimulating the protein synthesis that is required for the late phase of LTP. Indeed, we recently demonstrated that the synaptic enhancement induced by BDNF in hippocampal slices requires the synthesis of new proteins, perhaps in dendritic compartments (Kang and Schuman, 1996), and neurotrophin-stimulated protein synthesis has been observed in isolated cultured hippocampal neurites (Crino and Eberwine, 1996).

At what time are neurotrophins released during LTP? Our experiments with the function-blocking TrkB antiserum indicate a noticeable difference between antiserum-treated and control slices as early as 15 min following LTP induction by either pairing or TBS, suggesting release at or around the time of induction. In addition,

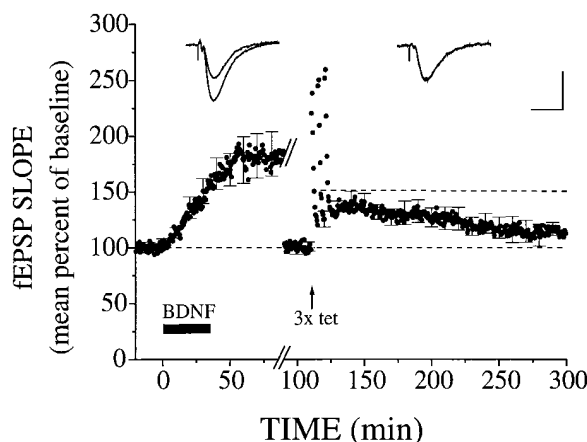


Figure 8. Prior Potentiation by BDNF Reduces Late-Phase LTP

Ensemble average of six experiments showing mean percentage of baseline values of field EPSP slopes before and after application of BDNF (50 ng/ml) and before and after application of three trains of 100 Hz stimulation to induce late-phase LTP. The amount of potentiation elicited by 100 Hz stimulation in these experiments was significantly less than that observed in control slices ($p \leq 0.05$; see Figure 7) but not significantly different from TrkB Ab-treated slices (Figure 7). Mean field EPSP slopes were 0.11 ± 0.01 mV/ms and 0.19 ± 0.03 mV/ms before and 80–90 min after BDNF application, respectively. Mean field EPSP slopes were 0.15 ± 0.01 mV/ms and 0.18 ± 0.02 mV/ms before and 170–180 min after LTP induction, respectively.

TrkB antisera-treated slices also showed slightly, although not significantly, diminished LTP within 10–20 min following the late-phase induction protocol. Thus, these data would suggest that the time of induction is an important period of neurotrophin release. The experiments using the TrkB-IgG fusion proteins, however, indicate that TrkB signaling is required for up to 1 hr following L-LTP induction. Taken together, these observations suggest that the induction of L-LTP either results in the sustained release of TrkB ligands or continuous signaling by ligands released at earlier times. Since the neurotrophins are secreted in both a regulated and constitutive manner (Bloch and Thoenen, 1995), it is possible that the particular patterns of synaptic activity associated with TrkB-dependent LTP result in the regulated release of BDNF or other TrkB ligands. The continued dependence of LTP on TrkB ligands during the enduring phase of the synaptic enhancement, which is not typically associated with increased neuronal activity, raises the possibility that sustained release of neurotrophic factors may participate in the maintenance of L-LTP. Current studies examining the spatial and temporal patterns of neurotrophin release will indicate whether such a scenario is possible.

What is the molecular basis of the demonstrated dependence of late-phase LTP on intact TrkB function? The maintenance of L-LTP involves new protein synthesis and perhaps cAMP-mediated gene expression (Frey et al., 1988, 1993; Impey et al., 1996; Nguyen et al., 1994). Although it has not been clearly demonstrated in vertebrate models of long-term plasticity, these protein synthesis-dependent processes result in morphological changes and growth of new synaptic connections in

Aplysia (Bailey and Chen, 1983; Nazif et al., 1991). Since the TrkB ligand BDNF requires local protein synthesis to enhance synaptic strength in the adult hippocampus (Kang and Schuman, 1996), it is possible that local protein synthesis involving TrkB ligands may contribute to L-LTP. Whether this same signaling pathway that results in clear changes in synaptic strength also alters synaptic structure, as has been observed in the developing nervous system (e.g., Cohen-Cory and Fraser, 1995; McAllister et al., 1995), remains to be determined.

Experimental Procedures

Antisera Preparation and Immunodetection

Rabbit polyclonal antisera were prepared according to standard immunological techniques, using the previously described extracellular domain of the TrkB receptor as an antigen (Philo et al., 1994). This antiserum was shown to be specific for the TrkB receptor (Yan et al., 1997). The ability of this antisera to functionally block the BDNF-induced phosphorylation of the TrkB receptor was tested in recombinant NIH3T3 cells, which express the full-length TrkB receptor. Cells were treated for 1 hr at 37°C with media containing either 10% preimmune serum, 10% anti-TrkB antiserum, or no antiserum, in the absence or presence of 50 ng/ml BDNF. After treatment, the cells were lysed, and TrkB receptors were immunoprecipitated and examined by Western blot analysis using either anti-phospho Tyr antiserum or anti-Trk antiserum, as previously described (Barnea et al., 1996). In experiments using hippocampal slices, following treatment with the TrkB antiserum for the times specified in the text and then lysing, immunoprecipitation and Western blot analysis were conducted as described above. For immunodetection of TrkB Ab, hippocampal slices were fixed and sectioned (50 μ m) and then treated with a Cy3-conjugated goat anti-rabbit secondary antibody. Immunostained specimens were viewed with a Zeiss LSM 310 laser-scan confocal microscope through either a 10 \times or a 63 \times oil-immersion lens. Cy3 was excited at 543 nm. Images were recorded through standard emission filters with the same contrast and brightness settings for all experimental and control sections.

Slice Preparation

Hippocampal slices were prepared using a Stoelting tissue chopper from young (6- to 8-week-old) adult male Sprague-Dawley rats. Prior to electrophysiological recording, slices were stored for at least 1.5 hr on a Millipore membrane (#1) placed over a tissue culture dish containing oxygenated Ringer's solution. The slice was exposed to 95% O₂/5% CO₂ circulating in an enclosed chamber. For electrophysiological recordings, slices were submerged in a stream of ACSF (119 mM NaCl, 2.5 mM KCl, 1.3 mM MgSO₄, 2.5 mM CaCl₂, 1.0 mM NaH₂PO₄, 26.2 mM NaHCO₃, and 11.0 mM glucose) maintained at room temperature (22°C–25°C) and perfused with 95% O₂/5% CO₂. The remaining slices were stored in an interface chamber at room temperature.

Electrophysiology

Field or intracellular EPSPs measured in stratum radiatum or in CA1 pyramidal cells, respectively, were evoked by stimulation of the Schaffer collateral-commissural afferents (1 stimulus every 30 s). Extracellular recording electrodes were filled with 3 M NaCl; intracellular recording electrodes were filled with 2 M cesium acetate. K252a was dissolved in DMSO; the control experiments associated with the K252a experiments were exposed to an equivalent final DMSO concentration (0.01%). Tetanic stimulation was delivered at the test intensity in 1 s trains at 100 Hz, with four trains 30 s apart or three trains 5 min apart, respectively, for short-term and long-term LTP. TBS consisted of 10 bursts of stimuli, each of four pulses at 100 Hz, with an interburst interval of 200 ms. Pairing was accomplished by sustained depolarization (to 0 mV) of the intracellularly recorded neuron by direct current injection in conjunction with low frequency (1 Hz) stimulation of the test pathway for 30 s. We analyzed the initial slope of the field EPSP and the slope and amplitude of the

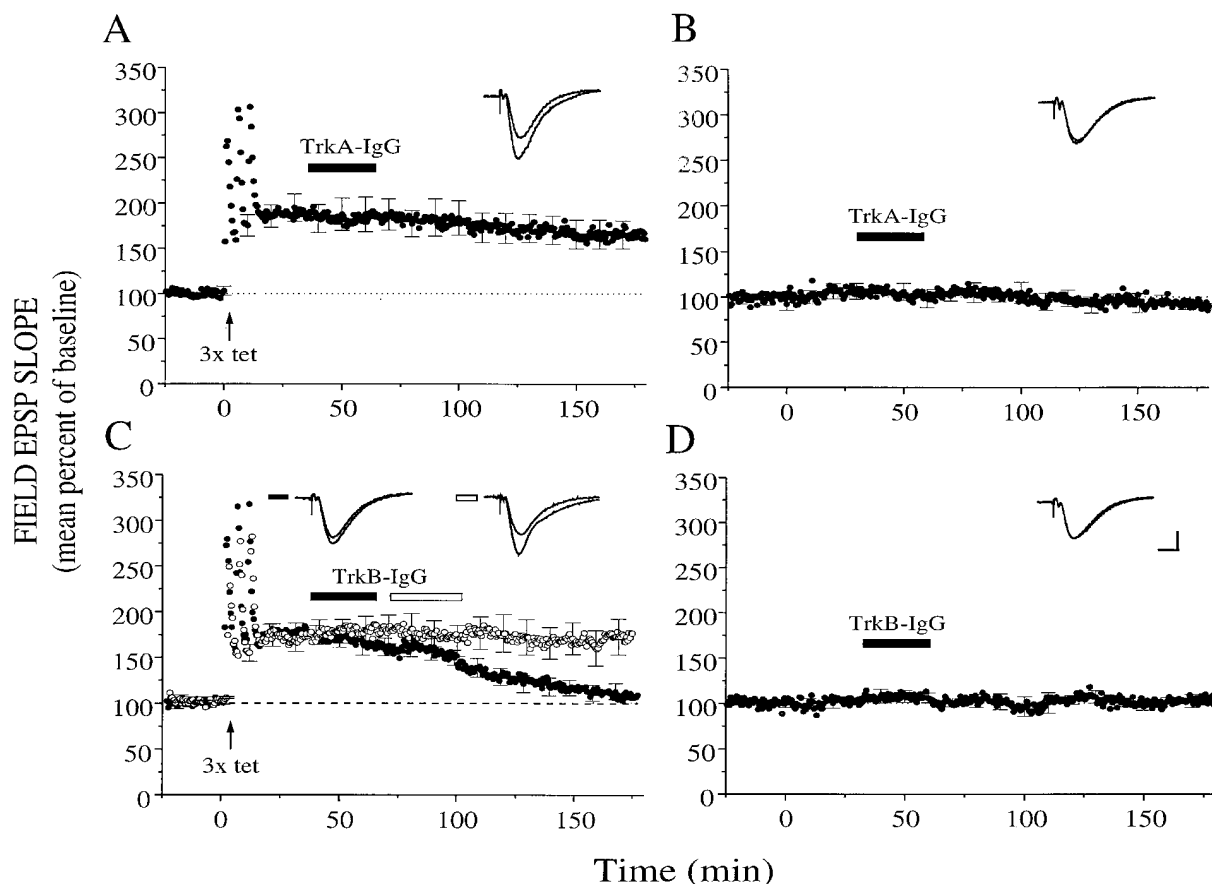


Figure 9. Effects of TrkA-IgG and TrkB-IgG on Late-Phase LTP

Ensemble averages showing mean percentage of baseline values of field EPSP slopes before and after LTP induction in slices treated for 30 min with either TrkA-IgG ([A] and [B]) or TrkB-IgG ([C] and [D]). Superimposed representative EPSPs were recorded 5 min before and 3 hr after LTP induction. Calibration bars, 1 mV and 20 ms.

(A) Effects of TrkA-IgG fusion proteins on L-LTP ($n = 6$). Mean field EPSP slope was 0.17 ± 0.01 mV/ms (mean \pm SEM) before and 0.27 ± 0.02 mV/ms 3 hr after LTP induction.

(B) Effects of TrkA-IgG fusion proteins on basal synaptic transmission in a nontetanized pathway ($n = 5$). Mean field EPSP slope was 0.18 ± 0.01 mV/ms before and 0.16 ± 0.02 mV/ms 3 hr after LTP induction of pathway 1 (tetanized).

(C) Effects of TrkB-IgG fusion protein, applied either 30–60 (closed bar; $n = 7$) or 70–100 (open bar; $n = 8$) min following induction, on L-LTP. For the 30–60 min application, the mean field EPSP slopes were 0.17 ± 0.01 mV/ms and 0.18 ± 0.01 mV/ms before and 3 hr after LTP induction, respectively. For the 70–100 min application, the mean field EPSP slopes were 0.15 ± 0.01 mV/ms and 0.25 ± 0.02 mV/ms before and 3 hr after LTP induction, respectively.

(D) Effects of TrkB-IgG fusion proteins on basal synaptic transmission in nontetanized pathway ($n = 6$). Mean field EPSP slope was 0.16 ± 0.01 mV/ms and 0.16 ± 0.01 mV/ms 3 hr after LTP induction of pathway 1 (tetanized).

intracellular EPSP. Ensemble averages were constructed using all data points, aligned with respect to the time of LTP induction or BDNF application. In some occlusion experiments, the stimulus strength was reduced following BDNF-induced potentiation in order to keep field EPSPs in a dynamic range; this had no effect on the subsequent ability to induce LTP. Error bars indicate standard error of the mean (SEM) calculated for the entire data set for a given time point. To assess statistical significance, paired t tests, comparing the average slope size for 10 min prior to LTP induction to either 50–60 or 170–180 min after LTP induction, were performed on non-normalized data. Between-group comparisons were made of percent of baseline values using unpaired t tests. Significance levels are as stated in the text; p values greater than 0.05 are designated as not significant (NS).

Blocking Antibody and Trk-IgG Experiments

Prior to electrophysiological recording, slices were individually incubated in single wells of a 24-well tissue culture plate in 200 μ l of Ringer's solution containing either a TrkB antiserum (1:7, 1:10, or

1:100) or an equivalent dilution of preimmune serum. Slices were incubated in the antiserum or preimmune serum for 1.5–2 hr prior to recording. Slices were then transferred to a recording chamber and perfused with normal Ringer's solution for \sim 30 min prior to LTP induction (Kang et al., 1996).

The Trk-IgG fusion proteins were stored at 4°C. Two independent pathways were stimulated in a single slice. Following LTP induction in a single pathway, Trk-IgG molecules were introduced into the ACSF superfusate at the dilutions indicated in the text for 30 min. A two-site ELISA assay was used to quantitate the percent intact TrkA- and TrkB-IgG. Following perfusion with either TrkA-IgG or TrkB-IgG for 30 min, hippocampal slices were flash frozen and then processed using the two-site ELISA assay as previously described (Cabelli et al., 1997).

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